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Appearance of Virus-specific DNA in Mammalian Cells following Transformation by Rous Sarcoma Virus

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To detect Rous sarcoma virus-specific DNA in mammalian cells, we have measured the capacity of unlabeled cell DNA to accelerate the reassociation of labeled double-stranded DNA synthesized by the Rous sarcoma virus RNA-directed DNA polymerase. Two populations of double-stranded polymerase products are identified by their reassociation kinetics and represent approximately 5% and 30% of the viral 70 S RNA genome. Using two strains of Rous sarcoma virus and four lines of transformed mammalian cells, we found two copies of DNA homologous to both DNA populations in Rous sarcoma virus-transformed rat and mouse cells, but not in normal cells. The Rous sarcoma virus-like DNA can be demonstrated in the non-repeated fraction of transformed cell DNA and in nuclear DNA. The results are supported by evidence that the techniques employed detect the formation of extensive well-matched duplexes of cell DNA and viral polymerase products.

1. Introduction

We have previously reported that the RNA-directed DNA polymerase associated with Rous sarcoma virus preferentially copies about 5% of the 70 S RNA genome into double-stranded DNA; a minor fraction of the double-stranded DNA is transcribed from about 30% of the genome (Varmus *et al.*, 1971). These populations of DNA molecules are characterized and prepared by their reassociation kinetics; the former is termed rapidly-reassociating DNA and the latter slowly-reassociating DNA. DNA from a variety of avian cell types contain RSV†-specific nucleotide sequences as shown by a capacity to accelerate the reassociation of virus-specific polymerase products. Ten to fifteen copies of sequences homologous to rrDNA and srDNA have been detected in both normal and RSV-transformed chick cells (Varmus *et al.*, 1972).

† Abbreviations used: RSV, Rous sarcoma virus; rrDNA, rapidly-reassociating DNA; srDNA, slowly-reassociating DNA; SR-RSV, Schmidt-Ruppin strain of RSV.

These observations provide confirmation of hypotheses that postulate the presence of tumor virus genes in normal cells (Huebner & Todaro, 1969; Todaro & Huebner, 1972). However, they cannot be interpreted as evidence against the "provirus" hypothesis that transformation is accompanied by an increased number of RNA tumor virus-specific sequences in cell DNA (Temin, 1963, 1972), since a small increase might not have been detectable in the face of multiple copies in normal cells, and since the polymerase products used as probes were not representative of the entire viral genome. Having been unable to transcribe the complete 70 S genome uniformly into double-stranded DNA under a variety of conditions, or with purified RSV polymerase (Varmus *et al.*, 1971; Taylor *et al.*, 1972), we have tested the provirus hypothesis by asking whether RSV-specific DNA could be measured following RSV-induced transformation of mammalian cell lines which normally do not contain RSV-specific nucleotide sequences. We found that established lines of mouse and rat cells, initially devoid of RSV-specific DNA, acquired approximately two copies per cell of DNA homologous to at least 25% of the RSV genome as a result of transformation by B77 or Schmidt-Ruppin strains of RSV.

2. Materials and Methods

(a) *Viruses and cells*

Avian sarcoma virus B77 (subgroup C) (Thurzo *et al.*, 1963) and the Schmidt-Ruppin strain of RSV (SR-RSV, subgroup A) were grown in infected chick embryo fibroblasts (Kimber Farms, Berkeley, Calif.) and purified as previously described (Bishop *et al.*, 1970).

Normal rat kidney (NRK) cells and 3T3 cells (line A31) from BALB/c mice were a gift of Dr G. Todaro. Lines of these mammalian cells transformed by avian sarcoma viruses were obtained by infecting monolayers of 10^6 cells with approximately 10^6 focus-forming units of B77 or SR-RSV (subgroup D) in the presence of 2 μg of polybrene/ml (Aldrich) (Toyoshima & Vogt, 1969). The infected cells were transferred at 3-day intervals, and after the third transfer they were suspended in nutrient agar according to published techniques (Friis *et al.*, 1971). Only virus-infected cultures yielded actively growing colonies in agar suspension. These colonies were picked and grown into cell lines designated as B77/NRK, SR/NRK, B77/3T3, and SR/3T3. The cells were morphologically transformed but, like most other mammalian cells transformed by RSV, did not produce virus detectable by labeling with [^{3}H]uridine or by focus formation on chick cells. The infecting virus was rescued from B77/NRK, SR/NRK and B77/3T3 by cocultivation of the transformed mammalian cells with normal chick fibroblasts. SR/3T3 cells did not yield virus under these conditions but did contain group-specific antigens of the avian RNA tumor viruses. Normal rat kidney or 3T3 cells were negative for virus in cocultivation tests and did not contain avian tumor virus group-specific antigens.

Normal and transformed mouse cells were grown in monolayer culture in Dulbecco's modified Eagle's Medium (Grand Island Biological Company) supplemented with 10% calf serum. Rat cells were grown in Medium 199 (Grand Island Biological Company) supplemented with tryptose phosphate broth and 5% calf serum.

(b) *Preparation of virus-specific double-stranded DNA*

Labeled double-stranded DNA homologous to 70 S viral RNA was prepared with Nonidet-P40 (Shell Chemical Co.) detergent-treated virus in the presence of 10 mM-$MgCl_2$, 100 mM-Tris·HCl (pH 8·1) and 2% β-mercaptoethanol (Garapin *et al.*, 1970). For synthesis of rrDNA, [^{3}H]dCTP (Schwarz-Mann; 22·6 Ci/mmol), [^{3}H]dATP (New England Nuclear; 5·73 Ci/mmol), [^{3}H]dGTP (Amersham-Searle; 13·4 Ci/mmol) and [^{3}H]TTP (Schwarz-Mann; 13·5 Ci/mmol) were present at concentrations of 10 μM, and the specific activity of the DNA product was $2{\cdot}5 \times 10^7$ cts/min/μg. For synthesis of srDNA, precursors included [^{3}H]TTP (at $3{\cdot}2 \times 10^{-5}$ M) and unlabeled dGTP, dCTP and dATP (at $8{\cdot}0 \times 10^{-5}$ M); the

specific activity of the product was 5×10^6 cts/min/μg. After 18-h incubations at 37°C, the reactions were terminated with 0·5% sodium dodecyl sulfate and the DNA products prepared by pronase digestion (500 μg/ml), 2 phenol extractions (room temp), ethanol precipitation, and pancreatic ribonuclease digestion (100 μg/ml, 37°C, 0·003 M-EDTA, for 1 h). Single-stranded and double-stranded DNA were separated by batch elution from hydroxyapatite powder (DNA grade, Biorad) (Bernardi, 1971; Fanshier *et al.*, 1971). DNA of the higher specific activity was used without further fractionation and was therefore composed principally of rrDNA, reassociating with a $C_0t_{1/2}$ of 1 to $2{\cdot}5 \times 10^{-3}$ mol s/l and representing about 5% of the RSV genome. srDNA was separated from the bulk of the double-stranded product of lower specific activity on the basis of its reassociation kinetics as outlined previously (Varmus *et al.*, 1971). This population of DNA molecules had a $C_0t_{1/2}$ of 1·1 to $1{\cdot}3 \times 10^{-2}$ mol s/l and is assumed to represent about 30% of the RSV genome. The computation of genomic representation from $C_0t_{1/2}$ values is based upon the demonstrated relation between sequence complexity and reassociation kinetics (Britten & Kohne, 1968). The reassociation of all labeled DNA preparations used in these experiments was analyzed in the absence of cell DNA and conformed to previous results (Varmus *et al.*, 1971). An apparent increase in the $C_0t_{1/2}$ in the presence of unrelated cell DNA observed in some experiments proved to be an artifact of the hydroxyapatite assay and was not observed when larger amounts of hydroxyapatite were used.

(c) *Preparation of cellular DNA*

Cellular DNA was purified by a modification of the method of Berns & Thomas (1965). Cells were lysed with 0·4% sodium dodecyl sulfate, in buffer containing 0·05 M-Tris·HCl (pH 8), 0·01 M-EDTA and 0·1 M-NaCl, digested overnight with pronase (100 μg/ml), extracted twice at room temperature with buffer-saturated phenol, ethanol precipitated, treated overnight in 0·01 M-EDTA with pancreatic ribonuclease (Sigma) (100 μg/ml), and again extracted twice with phenol. The DNA was then dialyzed for several days against 0·1 × SSC (SSC is 0·15 M-NaCl and 0·015 M-sodium citrate) and sheared to a length of approximately 150 to 200 nucleotide pairs at 50,000 lb/in^2 in a pressure cell designed by the American Instrument Company (Silver Spring, Maryland). After chloroform extraction and ethanol precipitation, the DNA was dissolved in a small volume of 0·003 M-EDTA and its concn determined by optical density at 260 nm in a Gilford spectrophotometer. (The ratio of OD_{260}/OD_{280} was invariably greater than 1·85.) Salmon sperm and calf thymus DNA were obtained commercially (Sigma) and repurified. Heavy-density chick DNA was made by growing SR-RSV transformed chick cells for 5 days in 5 μg of 5-bromodeoxyuridine/ml (BrdUrd, Calbiochem) and by separating it after DNA extraction from DNA of intermediate densities in CsCl equilibrium density step-gradients (Brunk & Leick, 1969). Gradients were centrifuged for at least 36 h at 33,500 revs/min at 25°C and fractions were assayed for A_{260} and, when indicated, acid-precipitable cts/min. Nuclei were prepared according to a modification of the method published by Penman (1966). Cells swollen for 10 min at 4°C in 0·001 M-NaCl, 0·00015 M-$MgCl_2$, 0·001 M-Tris·HCl (pH 7·4) were disrupted with 15 strokes in a Dounce homogenizer. Cell breakage was monitored by phase microscopy. Nuclei were pelleted at 2000 revs/min, washed twice with a mixture of 0·87% Nonidet-P40 and 0·44% sodium deoxycholate, dispersed in distilled water, and lysed for DNA extraction with 0·4% sodium dodecyl sulfate.

(d) *DNA reassociation*

DNA reassociation was studied by incubating mixtures of labeled virus-specific DNA and unlabeled cell DNA at 68°C in 0·40 M-phosphate buffer (equimolar NaH_2PO_4 and Na_2HPO_4) after denaturation of nucleic acid at 100°C in 0·003 M-EDTA. Samples were usually assayed by fractionation on hydroxyapatite; unlabeled DNA in eluates was measured spectrophotometrically and labeled DNA by trichloroacetic acid precipitation and counting in a Beckman scintillation counter with toluene-Liquifluor (New England Nuclear Co.). Results were plotted against the product of initial concn and time (C_0t), with correction for Na^+ concn in the annealing mixture (Britten & Smith, 1970), according to the convention established by Britten & Kohne (1968). When secondary structure was analyzed with single-strand-specific nuclease from *Aspergillus oryzae* (S_1 nuclease)

(Sutton, 1971), the annealing reactions contained 0·6 M-NaCl, 0·02 M-Tris·HCl, pH 7·0. Samples were diluted into a buffer containing 0·1 M-sodium acetate (pH 4·5), 0·003 M-$ZnCl_2$, 0·3 M-NaCl, and 10 μg heat-denatured calf thymus DNA/ml, and were incubated for 3 h at 50°C with and without S_1 nuclease, precipitated with trichloroacetic acid and counted. Nuclease resistance was plotted against C_0t. Both the hydroxyapatite and S_1 nuclease assayed were monitored routinely with DNA's of known secondary structure, and the necessary minor corrections of the raw data were made in accordance with these standards. Computations of copy numbers were based upon the estimated molecular complexity of double-stranded polymerase products (10^6 for rrDNA, 6×10^6 for srDNA) (Varmus *et al.*, 1971), reported values for the molecular weights of diploid complements of rat and mouse cell DNA (approximately 4×10^{12}) or chick cell DNA (approximately $1{\cdot}6\times10^{12}$) (Shapiro, 1968), and the arithmetic outlined by Gelb *et al.* (1971*a*). The ratio of viral sequences to diploid complements of cell DNA in a reassociation experiment was determined from the cts/min per ml of the labeled virus-specific DNA and from the optical density of the cell DNA. This ratio was used to compute the number of copies of virus-specific DNA in each diploid cell required to produce an observed accelerated reassociation of the labeled DNA. Thus, a reaction might contain one set of B77 ^{3}H-labeled srDNA sequences for each diploid complement of B77/3T3 DNA; a 3-fold reduction in the $C_0t_{1/2}$ of the ^{3}H-labeled srDNA (in comparison with its $C_0t_{1/2}$ in the presence of an equivalent concn of salmon sperm DNA) would indicate that 2 copies of viral sequences in each diploid genome caused a 3-fold increase in the effective concn of srDNA. In experiments reported here, B77 polymerase products were used to test B77-transformed cells and SR-RSV products to test SR-RSV-transformed cells. Unpublished observations, however, indicate considerable homology between the DNA's synthesized by the two strains. The products of one strain are in fact capable of detecting RSV-like DNA in cells transformed by the other.

3. Results

(a) *Validation of the reassociation kinetics assay for viral sequences*

Reconstruction experiments using mixtures of known amounts of unlabeled SV40 virus and cellular DNA have established that the number of sequences homologous to labeled SV40 DNA in the unlabeled DNA pool can be accurately assessed by the effect of unlabeled DNA upon the reassociation kinetics of the labeled DNA (Gelb *et al.*, 1971*a*). To substantiate further the reliability of this technique as applied to detection of RSV-specific DNA, we carried out a series of experiments to determine (1) whether true hybrids of viral DNA and cell DNA were formed when reassociation was accelerated; (2) whether these hybrids exhibited errors in base-pairing as measured by the kinetics of thermal denaturation; and (3) whether significant single-stranded viral DNA tails were present in the hybrids. The logistics of these experiments required that 10 to 20×10^3 cts/min of RSV-specific DNA be annealed to several milligrams of cell DNA; therefore they were carried out principally with chick cell DNA, previously shown to contain 10 to 15 copies of viral sequences per cell, rather than with RSV-transformed mammalian DNA which, as shown below, contains only two copies per cell.

To demonstrate the formation of hybrids between cell DNA and DNA synthesized by RSV polymerase, we measured the shift in density of ^{3}H-labeled polymerase product in CsCl equilibrium gradients after reassociation with high-density DNA from chick cells grown in the presence of 5-bromodeoxyuridine. SR-RSV transformed chick cells were grown for five days with 5 μg of BrdUrd/ml; a portion of these cells was also labeled with $^{32}PO_4$ (100 μCi/ml). After extraction, about 75% of the DNA (HH-DNA) banded in a high-density region (1·740 to 1·760) of a CsCl equilibrium gradient and contained, therefore, brominated bases in both strands. This DNA was

pooled from several gradients and sheared at 50,000 lb/in^2. The remainder of the DNA banded at an intermediate density and was discarded. Double-stranded [^{3}H]-DNA synthesized by RSV polymerase bands in CsCl at the same density as normal chick DNA (LL-DNA, 1·700). After reassociation of ^{3}H-labeled rrDNA in the presence of sufficient HH-DNA to accelerate the reaction fourfold, the vast majority of polymerase product bands at an intermediate density (Fig. 1). This result indicates that the acceleration of duplex formation is due to formation of hybrids between cellular DNA and ^{3}H-labeled virus-specific DNA.

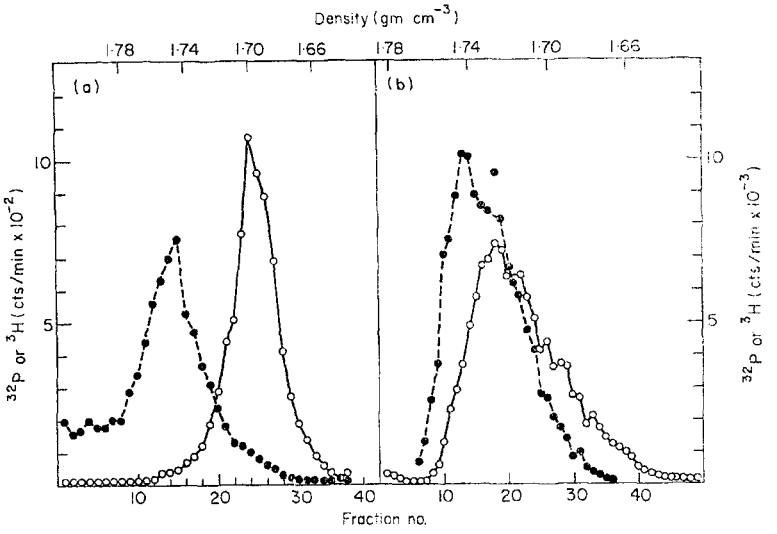

FIG. 1. Demonstration of cell DNA–viral DNA hybrid after reassociation.

^{3}H-labeled double-stranded DNA synthesized by SR-RSV associated polymerase (spec. act. $2{\cdot}5 \times 10^7$ cts/min/μg, 3·2 ng/ml) was denaturated and incubated at 68°C for 22 h in 0·40 M-phosphate buffer in the presence of 1·4 mg of sheared ^{32}P-labeled HH-DNA/ml (see Results, section (a)) prepared from BrdUrd treated SR-RSV-transformed chick cells as described in the text. After incubation, the DNA was eluted from hydroxyapatite and the reassociated DNA (78% of the [^{3}H]DNA, 70% of the [^{32}P]DNA) was centrifuged (33,500 revs/min, 25°C, 66 h) in a CsCl density equilibrium gradient, panel (b). Centrifugation of a similar mixture of ^{32}P-labeled HH-cell DNA and ^{3}H-labeled virus-specific DNA which was not denatured or hybridized, panel (a). —●—●—, ^{32}P-labeled HH-DNA; —○—○—, [^{3}H]DNA.

To assess the fidelity of base-pairing in these hybrids of cell and RSV DNA, we compared the thermal denaturation of the hybrid duplexes (formed under conditions in which only about 25% of the viral DNA would self-anneal) and of labeled RSV-specific DNA reassociated in the absence of cell DNA. Samples of DNA were incubated for 15 minutes at each of several temperatures in 0·01 M-phosphate buffer. At the conclusion of each incubation the samples were removed, chilled in ice-water and assayed on hydroxyapatite. As shown in Figure 2, both rrDNA and srDNA melt sharply whether self-annealed or annealed with chick DNA. The T_m for both self-associated DNA and for cell-product hybrids is about 70°C under these conditions, suggesting there is little, if any, mismatching of base pairs. (Control experiments showed that the melting profile of the polymerase product was not altered after reassociation.) A similar experiment performed with B77-transformed 3T3 cell DNA and B77 srDNA, under conditions in which about one third of the viral product

self-anneals and two thirds anneal with cell DNA, demonstrates that hybrids formed with mammalian cell DNA have the same thermal stability as the srDNA self-annealed in the presence of calf thymus DNA (data not shown).

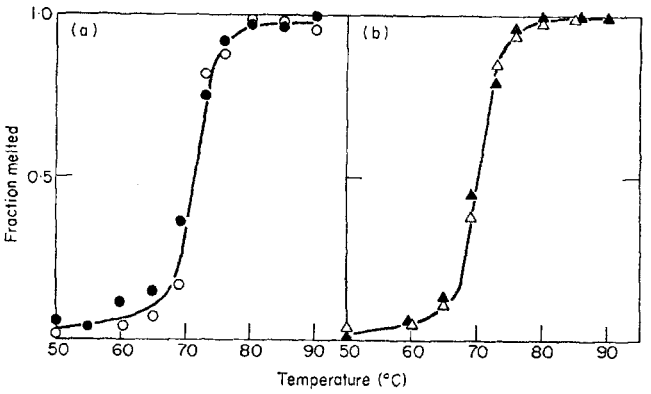

FIG. 2. Thermal denaturation of rrDNA, srDNA and hybrids formed with cell DNA.
^{3}H-labeled rrDNA and ^{3}H-labeled srDNA (spec. act. 5×10^6 cts/min/μg, approximately 12,000 cts/min of each) were reassociated with sufficient chick embryo fibroblast DNA to accelerate the reannealing of the viral products about 4-fold. The reannealed materials were eluted from hydroxyapatite in 0·40 M-phosphate buffer and dialyzed against 0·01 M-phosphate buffer. Samples of the hybrid populations and the starting rrDNA and srDNA preparations were equilibrated at several temperatures for 15 min in a circulating water bath. The samples were then chilled in an ice-water bath and subsequently assayed with hydroxyapatite for extent of double-strandedness. Results are plotted against temperature. (a) (○) rrDNA; (●) rrDNA–chick DNA hybrid. (b) (△) srDNA; (▲) srDNA–chick DNA hybrid.

Since the hydroxyapatite assay scores molecules eluting at the higher phosphate concentration as completely double-stranded, an attempt was made to assess the amount of genuine duplex in these molecules by subjecting them to degradation with a single-strand-specific S_1 nuclease prepared from *Aspergillus oryzae* (Sutton, 1971). Double-stranded DNA's synthesized by RSV polymerases were generally 90 to 100% resistant to digestion by S_1 nuclease (Leong *et al.*, 1972). Duplex molecules formed during reassociation experiments and eluted from hydroxyapatite in 0·40 M-phosphate buffer consistently showed about 80% resistance to digestion, indicating the presence of some single-stranded tails and/or loops in the reformed duplexes. The S_1 nuclease assay was also used to judge the extent of reassociation during kinetic experiments. An example is shown in Figure 3. The rate of reannealing of [^{3}H]DNA synthesized by RSV polymerase is shown to be accelerated several-fold by chick DNA in comparison to salmon sperm DNA. The degree of acceleration—$C_0 t_{1/2}$ reduced from $1{\cdot}7 \times 10^{-3}$ to $2{\cdot}8 \times 10^{-4}$ mol s/l—is consistent with the presence of about ten copies of RSV sequences per diploid chick cell. The copy number and $C_0 t_{1/2}$ values are similar to those previously reported with the hydroxyapatite assay (Varmus *et al.*, 1972). However, despite the ease of carrying out the S_1 nuclease assay, it requires considerable expenditure of DNA for each point in a kinetic experiment and in our hands was somewhat less precise than the highly reproducible hydroxyapatite assay. Therefore, it was used infrequently in experiments with mammalian DNA's (see Fig. 9).

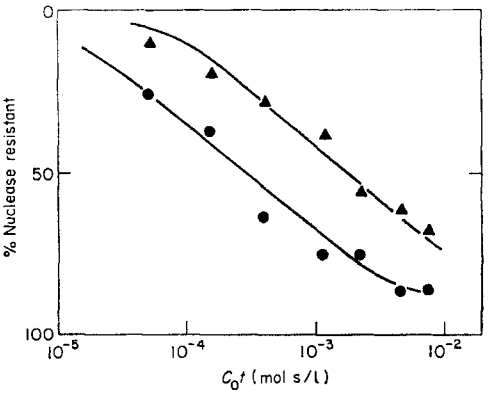

FIG. 3. Measurement of RSV-specific DNA in chick cells with S_1 nuclease assay.

[^{3}H]DNA ($2{\cdot}5\times10^7$ cts/min/μg; 1·9 ng/ml) synthesized by the B77 RSV polymerase was heat-denatured and incubated at 68°C in 0·6 M-NaCl with 2 mg of salmon sperm DNA/ml (—▲—▲—) or DNA from B77-transformed chick cells (—●—●—). Samples were removed periodically and assayed for extent of duplex formation by incubation with single-strand-specific S_1 nuclease from *A. oryzae* as described in Materials and Methods.

(b) *Detection of RSV-specific rrDNA sequences in transformed rat and mouse DNA's*

To obtain maximum sensitivity for detection of small numbers of RSV-specific sequences, double-stranded products of B77 and SR-RSV polymerases were synthesized with four labeled deoxyribonucleoside triphosphate precursors. The specific activity of this DNA was $2{\cdot}5\times10^7$ cts/min/μg. As we have shown previously, about 85% of the product reassociated rapidly (rrDNA); small amounts of this highly-labeled product were made and it was not fractionated further. When labeled DNA synthesized by B77 polymerase was denatured and reassociated in the presence of DNA extracted from normal 3T3 cells (Fig. 4), the kinetics were identical to those

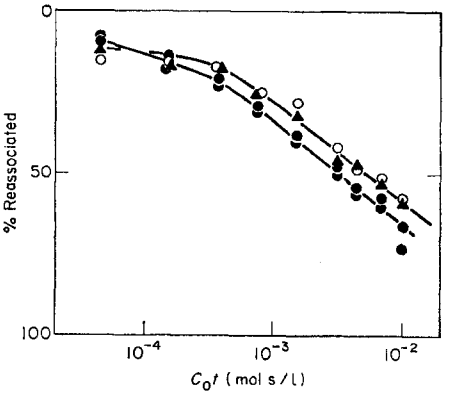

FIG. 4. Detection of B77-RSV-specific rrDNA in B77-transformed 3T3 cell DNA.

^{3}H-labeled double-stranded DNA (spec. act. $2{\cdot}5\times10^7$ cts/min/μg, 2·8 ng/ml) synthesized by B77 virus-associated DNA polymerase was denatured by heating at 100°C for 2 min and incubated at 68°C on 0·40 M-phosphate buffer with 2 mg of unlabeled DNA/ml from salmon sperm (▲), normal 3T3 cells (○), or B77/3T3 cells (●). Samples were removed from the incubation mixture at several time points, diluted into chilled 0·01 M-phosphate buffer, and assayed for secondary structure with hydroxyapatite. Results are plotted as a function of C_0t values computed for the labeled DNA polymerase product.

seen in the presence of salmon sperm DNA, which served as a viscosity control (Wetmur & Davidson, 1968). DNA from B77-transformed 3T3 cells, however, accelerated the reassociation. In this experiment the ratio of viral gene copies to cellular genomes was approximately 5 and the acceleration was 1·45; this indicates that about 2·25 copies of the viral genes under study (about 5% of the genome) are present per diploid cell, following transformation by RSV. Similar results were obtained with SR-RSV-transformed 3T3 cells and with transformed normal rat kidney cells using virus-specific rrDNA probes (Table 1).

TABLE 1

RSV-specific sequences in mammalian DNA

DNA source	Copy number per diploid cell		Present in unique-sequence DNA	
	rrDNA	srDNA	rrDNA	srDNA
3T3 cells	0	0	0	0
B77/3T3 cells	1·5, 2·3	1·8	+	+
B77/3T3 nuclei	N.T.†	2·0	N.T.	N.T.
SR/3T3 cells	2·5	1·4	+	+
NRK‡ cells	0	0	0	0
B77/NRK cells	N.T.	2·0, 1·1	+	+
SR/NRK cells	1·1	2·8	+	+

† N.T., not tested.
‡ NRK, normal rat kidney.

Results presented in Figure 4 and Table 1 show that approximately two copies of viral genes are found in the complete genome from RSV-transformed cells. This suggested that we could enrich cell DNA for virus-specific sequences and accentuate the difference between normal and transformed cells by removing reiterated DNA on the basis of its reassociation kinetics (Britten & Kohne, 1968). The data indicated that dilute labeled virus-specific DNA would reassociate at an accelerated rate concomitant with unique-sequence DNA from transformed cells, whereas its reassociation would be unaffected by the presence of untransformed cell DNA. Unique-sequence cell DNA was prepared by incubating sheared DNA to C_0t values (500 to 800 mol s/l) at which all of the reiterated, but little of the unique-sequence DNA would be reannealed. The unique sequence DNA was eluted from hydroxyapatite in 0·16 M-phosphate buffer, dialyzed, and concentrated by ethanol precipitation. Double-stranded viral [^{3}H]DNA was then reannealed with unique-sequence cell DNA at low ratios of viral to cellular DNA's. As expected, reassociation of viral DNA is coincident with the reassociation of unique-sequence DNA only when DNA from transformed lines is used (Fig. 5). Reassociation of the viral product is unaffected by the reassociation of unique-sequence DNA from calf thymus or from normal 3T3 cells (Fig. 5) or by the reassociation of reiterated sequences from transformed cell DNA (data not shown). Parallel experiments with transformed normal rat kidneys cells again gave identical results (Table 1). The findings indicate that the limited numbers of viral sequences under test in these experiments are all present in unique-sequence DNA from transformed rat and mouse cells.

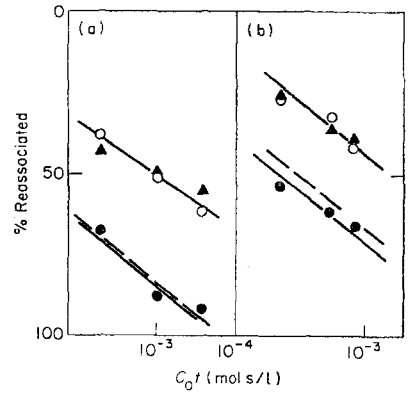

FIG. 5. Annealing of RSV-specific rrDNA with unique-sequence mouse cell DNA.

Unique-sequence DNA from calf thymus, normal 3T3 cells, and B77 or SR-RSV-transformed 3T3 cells was prepared by reassociating sheared DNA to C_0t values of 800 mol s/l, and fractionating the DNA on hydroxyapatite. Following dialysis and ethanol precipitation, the non-reassociated (unique) fractions were incubated (3 mg/ml) to cell C_0t values greater than 10,000 in the presence of denatured double-stranded [^{3}H]DNA (0·23 ng/ml, spec. act. $2{\cdot}5\times10^7$ cts/min/μg) synthesized by RSV polymerase. Samples were eluted from hydroxyapatite and fractions were assayed for optical density (260 nm) and for acid-precipitable radioactivity to determine the reassociations of cell and virus-specific DNA, respectively. (a) Reannealing of B77-specific [^{3}H]DNA with unique calf thymus (▲), 3T3 (○), and B77/3T3 (●) DNA. (b) Reannealing of SR-RSV-specific [^{3}H]DNA with unique calf thymus (▲), 3T3 (○), and SR/3T3 (●) DNA. In both panels, the reassociation of cell DNA's (optical density) is indicated by a dashed line, and the results are plotted against C_0t for the virus-specific DNA.

(c) *Detection of RSV-specific srDNA sequences in transformed rat and mouse DNA's*

To increase the number of viral sequences measured in these experiments, we prepared large quantities of double-stranded [^{3}H]DNA (spec. act. 5×10^6 cts/min/μg, [^{3}H]TTP only labeled precursor) with B77 and SR-RSV polymerases and isolated the slowly reassociating DNA (srDNA) as previously described (Varmus *et al.*, 1971). The srDNA prepared from both virus strains reannealed with $C_0t_{1/2}$ values of about $1{\cdot}2\times10^{-2}$ mol s/l. According to our present estimates the complexity of this DNA is at least 6×10^6 daltons, and it represents, therefore, about 30% of the viral 70 S RNA genome, the molecular weight of which is approximately 10^7 (Robinson & Duesberg, 1968). Figure 6 and Table 1 demonstrate that approximately two copies of these sequences per diploid cell are again observed in 3T3 cells transformed by either virus strain, whereas uninfected cells lack DNA homologous to the RSV polymerase product.

The same result is obtained with RSV-transformed normal rat kidney cells (Fig. 7). In this experiment, srDNA was also incubated with DNA from RSV-transformed chick cells to demonstrate the several-fold greater effect of avian DNA upon the reassociation of RSV DNA. In accord with previous results, a diploid complement of chick DNA contains 16 copies of sequences represented in srDNA, whereas in this experiment the B77-transformed normal rat kidney cells appear to contain two copies of those sequences. Similarly, DNA from SR-RSV-transformed normal rat kidney cells demonstrates 2·8 copies of srDNA sequences (Table 1).

To magnify the difference between normal and RSV-transformed cells and to

estimate the extent of representation of srDNA sequences in transformed cells, experiments analogous to those illustrated in Figure 5 were carried out with srDNA and unique-sequence cell DNA. Figure 8 demonstrates that, as with rrDNA, the reassociation of srDNA is markedly accelerated with unique-sequence DNA from RSV-transformed but not from normal rat and mouse cells. However, despite high

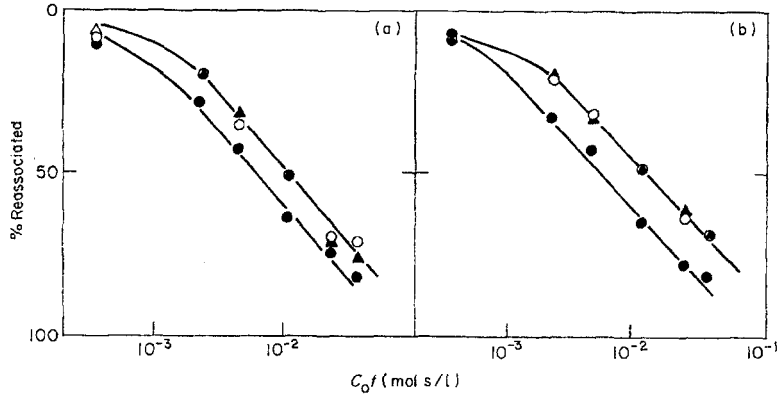

FIG. 6. Detection of RSV-specific srDNA in RSV-transformed mouse cells.
^{3}H-labeled srDNA (5×10^6 cts/min/μg) synthesized by B77 or SR-RSV polymerase was reassociated in the presence of 4 mg of DNA/ml from calf thymus (▲), normal 3T3 cells (○), or RSV-transformed 3T3 cells (●), and assayed with hydroxyapatite. (a) SR-RSV-specific srDNA (10·3 ng/ml) and SR/3T3 cell DNA; (b) B77-specific srDNA (9·3 ng/ml) and B77/3T3 cell DNA.

ratios of cell to virus-specific DNA, the percentage of reannealing of the srDNA consistently lagged behind the reannealing of unique-sequence cell DNA. Since absence of some of the srDNA sequences from transformed cell DNA should produce a flattened or biphasic curve, the observed lag probably represents an, as yet unexplained, artifact of the procedure.

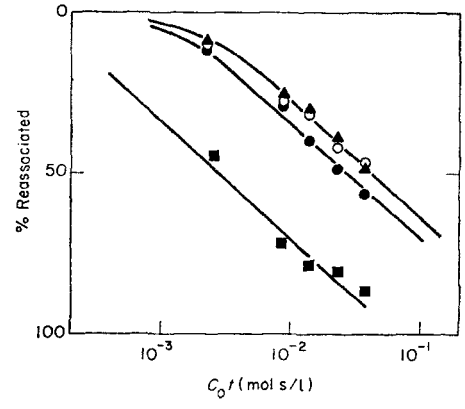

FIG. 7. Detection of B77 RSV-specific srDNA in B77-transformed rat and chick cells.
^{3}H-labeled srDNA (5×10^6 cts/min/μg) synthesized by the DNA polymerase associated with B77 strain was reannealed at 10 ng/ml in the presence of 2 mg of unlabeled DNA/ml from calf thymus (▲), normal rat kidney cells (○), B77/normal rat kidney cells (●), and B77-transformed chick cells (■) and assayed for reassociation with hydroxyapatite.

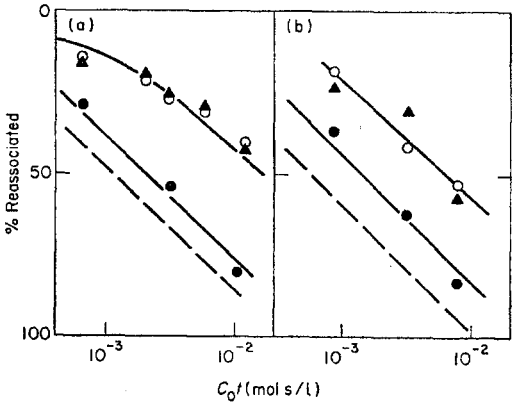

FIG. 8. Annealing of RSV-specific srDNA to unique-sequence cell DNA.

Unique-sequence DNA was prepared from several cell types as described in the legend to Fig. 5. ^{3}H-labeled srDNA (spec. act. 5×10^6 cts/min/μg, 2·3 ng/ml) was reannealed in the presence of 5·15 mg of unique-sequence DNA/ml from the indicated sources; reassociation kinetics of the cell DNA (measured by optical density of eluates from hydroxyapatite and summarized by dashed lines in each panel) and of the virus-specific DNA (measured by cts/min of ^{3}H in the same eluates and indicated by symbols and solid lines) are plotted as a function of the C_0t value for the virus-specific DNA. (a) B77-specific ^{3}H-labeled srDNA with calf thymus (▲), normal rat kidney (○), and B77/normal rat kidney unique-sequence DNA (●). (b) SR-RSV-specific ^{3}H-labeled srDNA with calf thymus (▲), 3T3 (○), and SR/3T3 unique-sequence DNA (●).

(d) *Localization of RSV-specific DNA in the nuclei of transformed mouse cells*

Since the experiments described in section (b) used DNA prepared from whole cells with extraction techniques designed to minimize loss of DNA molecules of small size, we tested nuclear DNA from B77-transformed mouse cells as a preliminary step in localizing the RSV-specific DNA within the cell. As shown in Figure 9, nuclear DNA

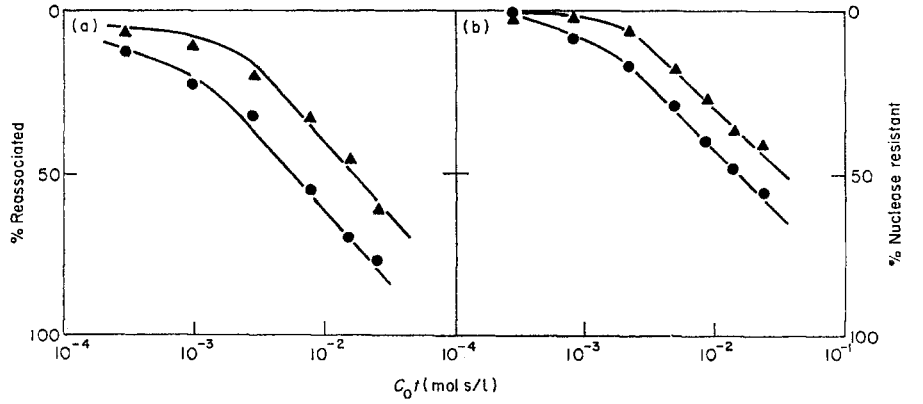

FIG. 9. Localization of B77-specific srDNA in nucleus of transformed mouse cells.

^{3}H-labeled srDNA (5×10^6 cts/min/μg, 7 ng/ml) synthesized by B77 polymerase was annealed with 4 mg of DNA/ml prepared as indicated in Materials and Methods from the nuclei of B77/3T3 cells (—●—●—). The same amount of B77-specific ^{3}H-labeled srDNA was reassociated in the presence of calf thymus DNA as a control (—▲—▲—). The extent of reassociation was assayed with hydroxyapatite (a) and with single-strand-specific S_1 nuclease (b).

from these cells is at least as effective as whole cell DNA (compare with Fig. 6(b)) in accelerating the reassociation of srDNA made by the B77 DNA polymerase. The results with whole cell DNA indicate 1·75 copies of srDNA sequences per diploid cell, and results with nuclear DNA demonstrate 2·0 copies per cell. Figure 9(b) also shows that the presence of RSV sequences in transformed mouse cell nuclei can be demonstrated by assaying reassociation with single-strand-specific nuclease, as well as with hydroxyapatite.

4. Discussion

(a) *Reassociation kinetics as a technique for measuring copies of RNA tumor virus-specific DNA in cells*

The experiments presented in Results section (a) show that the techniques described in this report detect the formation of stable hybrids between cell DNA and virus-specific DNA synthesized by RSV-associated DNA polymerase. These hybrids show little evidence of mismatched base pairs, as judged by thermal denaturation patterns, and only a small amount of single-stranded tails, as judged by resistance of the hybrids to a single-strand-specific nuclease. The data validate the specificity of this approach, which was first applied to detection of SV40 virus-specific sequences by Gelb *et al.* (1971*a*). Since SV40 DNA is well characterized and complete molecules are readily prepared from virus grown in productively-infected cells, computation of numbers of SV40-specific sequences in cell DNA is relatively simple; the accuracy of these computations is supported by reconstruction experiments (Gelb *et al.*, 1971*a*). The use of labeled DNA synthesized by RNA tumor virus polymerases introduces a number of uncertainties into these calculations: C_0t values must be based upon the specific activity of the DNA product, since insufficient quantities are made to determine optical densities; the double-stranded product is known to be moderately heterogeneous with respect to size (Taylor *et al.*, 1972) and complexity (Varmus *et al.*, 1971; Gelb *et al.*, 1971*b*) and often contains a small fraction of single-stranded tails; and the molecular complexity of the virus-specific DNA under study must be determined from the reassociation kinetics of the polymerase products measured in relation to standards of known complexity. Since these estimates of complexity indicate that the double-stranded products of the RSV polymerase represent a minority of the sequences present in the 70 S RNA genome (Varmus *et al.*, 1971), there is also an inherent limitation placed upon interpretation of the results. Despite these difficulties and the accompanying minor variations seen in Table 1, there are several indications that the copy numbers derived here and elsewhere are approximately correct. (1) Virus-specific DNA calculated to be present in two copies per diploid genome in transformed mammalian cells reassociates simultaneously with the unique-sequence DNA from those cells (cf. Fig. 5), but is unaffected by reannealing of reiterated DNA from the same cells. (2) When RSV-specific DNA is reannealed with unfractionated DNA from chick cells, which have been shown to contain 10 to 15 copies, virus-specific sequences reassociate about tenfold faster than unique copy cell DNA (Varmus *et al.*, 1972, and unpublished data). (3) Copy numbers for rrDNA and srDNA are very similar despite the introduction of the presumed sixfold difference in complexity into the computations.

(b) *Significance of results with RSV-transformed mammalian cells*

The observation that normal mouse and rat cell DNA's lack RSV-specific sequences and that transformation by RSV is accompanied by the addition of two copies of viral nucleotide sequences is consistent with the provirus mechanism for transformation by RNA tumor viruses proposed by Temin (1963), and with RNA–DNA hybridization with rat cell DNA recently published by Baluda (1972). The results suggest that in this situation the virus-associated RNA-dependent DNA polymerase serves an important biological function by providing DNA transcripts of at least some of the genome of the infecting virus.

At present it is not known whether all of the viral genome is present in the DNA of the transformed cell. (1) The most complex available double-stranded polymerase product (srDNA) appears to represent only about 30% of the genome. (2) Only a very small fraction of 70 S viral RNA is hybridized to transformed rat cell DNA in the RNA–DNA hybridization experiments reported by Baluda (1972). (3) Only partial annealing (20 to 25%) of probes representing the complete RSV genome—labeled 70 S RNA or single-stranded DNA synthesized in the presence of actinomycin D (Garapin *et al.*, 1973)—has been achieved with large excesses by RSV-transformed mouse DNA (Hansen *et al.*, unpublished data). (4) Although RSV-like virus can be rescued from transformed mammalian cells, Altaner & Temin (1970) have suggested that the altered efficiencies of plating of the rescued viruses may reflect changes in the viral genome.

(c) *Experimental approaches suggested by results with mammalian cells*

We have demonstrated (Fig. 9) that RSV-specific DNA is present in the nucleus of transformed mouse cells but more precise characterization of the physical state of the RSV genetic information is not presented in this report. Recent experiments, however, indicate that RSV-specific DNA is included in pieces of duplex DNA of greater than 30×10^6 molecular weight and that it is covalently linked to cell DNA containing reiterated sequences (Varmus & Martin, unpublished data). Since we have not established whether RSV RNA is completely represented in transformed mammalian cell DNA, and since the genome of the rescued virus may differ from the infecting genome, we are also investigating the possibilities that the RNA in the rescued virus may contain sequences newly acquired from the mammalian genome, or lack sequences present in the original transforming virus.

Attempts to study the relation of virus-specific RNA synthesis to transformation in the RSV-infected chick cell are complicated by the presence of the large amounts of virus-specific RNA required for viral replication. Since RSV-transformed mammalian lines show no evidence of RSV production, they provide an opportunity to study the regulation of transcription of genes presumably concerned with transformation events. Coffin & Temin (1972) have reported that RSV-transformed rat cells contain several-fold less virus-specific RNA than transformed chick cells. Our preliminary data confirm this observation and suggest that in some transformed lines extremely small quantities of virus-specific RNA may be present.

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